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C-Src and the epidermal growth factor receptor, both of which have been implicated in the genesis and progression of a number of human tumors, act synergistically in tumorigenesis. To gain further insights into the mechanism of c-Src synergy with the EGFR, stable cell lines containing various c-Src mutants and overexpressed *wt* EGFR were generated and examined for tumorigenic responses with the ultimate goal of determining which regions of c-Src are required for the functional and physical interaction with the receptor. Stable C3H10T1/2 mouse embryo fibroblasts overexpressing both the *wt* EGFR and either myristylation (M-), SH2 (S-) or kinase (K-) deficient c-Src, were created and assayed for colony formation in soft agar and tumor development in nude mice. The M- and S- c-Src potentiated EGF-dependent and independent tumorigenesis, while the K-/EGFR containing cell lines exhibited decreased growth in soft agar in the presence and absence of EGF and in tumor formation in nude mice, compared with double *wt* overexpressors. This biological effect correlated with the ability of K- c-Src to associate with the EGFR but it's inability to cause the phosphorylation of Tyr 845 on the receptor. K- c-Src also inhibited the anchorage independent growth of MDA-MB-468 and MCF-7 breast tumor cell transfectants.

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David A Tice 7/28/97

PI - Signature Date

TABLE OF CONTENTS

INTRODUCTION

The evidence linking proto-oncogenes to the induction or the progression of human malignancies is steadily increasing. Two of these proto-oncogenes, the epidermal growth factor receptor (EGFR), and cellular Src (c-Src) have been implicated in human breast carcinomas and linked to a worse prognosis or increased metastatic potential (1,2,3,4). Both proto-oncogenes are from different tyrosine kinase families, the EGFR is a member of the receptor tyrosine kinase family, while c-Src is of the non-receptor tyrosine kinase family. It has been reported that the EGFR gene and a closely related HER-2 gene are amplified in 25-30% of breast carcinomas (1). In addition, it was shown that amplification or activation of the EGFR and/or its family members were linked to the growth of several other human tumors including those of the prostate (5), ovary (6), colon (7), stomach (8), lung (9), bladder (10), brain (11), pancreas (5), larnyx (12), and esophagus (13). In direct tests of the transforming ability of the EGFR, activation of overexpressed EGFR by EGF was shown to be sufficient to transform NIH3T3 fibroblasts as observed in soft agar assays and in tumor formation in nude mice (14,15,16). An increase in c-Src kinase activity has also been implicated in a number of human malignancies, most notably breast (2), colon (17), brain (18), and head and neck carcinomas (19). In fact, 72 of 72 breast carcinomas studied exhibited an increase in cytosolic and membrane tyrosine kinase activity as compared to normal cellular controls, and c-Src kinase activity accounted for at least 70% of the cytosolic activity (2). Guy *et al.* (20) have shown that the presence of c-Src is required for mammary carcinoma development in transgenic mice expressing the polyomavirus middle T antigen. Several other recent studies have begun to show an interaction between the EGFR and c-Src both in physical association and signal transduction. *In vitro* binding was shown between endogenous c-Src and activated EGFR in a human breast cancer cell line MDA-MB-468 that overexpressed the EGFR (21). *In vitro* and *in vivo* complexes between c-Src and tyrosine phosphorylated HER-2/*neu* were seen in Neu-induced mammary tumors in transgenic mice that possessed an elevated c-Src kinase specific activity (22,23). An EGF-dependent activation of the Src family kinases was observed in mouse fibroblasts as well as colon and adrenal tumor cell lines that overexpress the EGFR (24). Additionally, work in our lab has recently shown that c-Src and the EGFR functionally interact, resulting in a potentiation of the transforming capacity of the EGFR as shown by growth in soft agar and tumor formation in nude mice (25). The mechanism of synergy between the two kinases has begun to be elucidated. Upon association of c-Src with the EGFR, two novel phosphorylations at Y-845 and Y-1101 on the receptor appear which correlate with increased phosphorylation of receptor substrates (25). These phenomenon have recently been correlated in breast tumor cell lines. In 5 of 5 breast tumor cell lines that overexpress both c-Src and EGFR, the two kinases associate with each other, the receptor in complex with c-Src gains both novel phosphorylations, Shc phosphorylation is elevated in response to EGF, and the cell lines exhibit a more aggressive tumor phenotype when compared to cell lines not overexpressing both c-Src and EGFR (26). This evidence is clearly pointing toward the further study of the synergism between the

EGFR and c-Src in the etiology of breast cancer so that specific points of interdiction can be recognized and attacked.

In my grant application I proposed to further dissect the mechanism of c-Src synergy with the EGFR and to use that understanding to block the mechanism in breast tumor cells. These studies were divided into four parts: First, a model cell line, C3H10T1/2 mouse embryo fibroblasts that overexpress EGFR and altered c-Src, will be used to determine which c-Src domains are necessary for EGF-induced mitogenesis and tumorigenesis. Assays used include ^{3}H thymidine incorporation, growth in soft agar, and tumor formation in nude mice. Second, the same model cells will be used to correlate the inhibitory activity of the c-Src mutants with the disappearance of complex formation between the EGFR and c-Src, increased phosphorylation of the receptor, and phosphorylation of receptor substrates. Third, isolated domains of c-Src will be used to disrupt the synergism between the EGFR and c-Src in breast tumor cell lines as determined in the same mitogenic and tumorigenic assays described above. And, 10T1/2 model cells and breast tumor cells will be examined for c-Src activation, and various mechanisms for that activation will be tested. If successful, new therapeutic and diagnostic tools for the treatment and recognition of breast cancer may result.

BODY

MATERIALS AND METHODS

Cell lines. The derivation and characterization of the clonal C3H10T1/2 murine fibroblast cell lines Neo (control), 5H (*wt* c-Src overexpressors), 2A (G2A myristylation deficient c-Src overexpressors), 155 (del155-157 SH2 mutant c-Src overexpressors), 430 (A430V kinase deficient c-Src overexpressors), NR (EGFR overexpressors), and 5HR (EGFR / *wt* c-Src double overexpressors) have been described previously (27, 28, 25). 2AR, 155R, and 430R cells were derived by infection of 2A, 155, and 430 cells respectively, with an amphotropic retrovirus encoding human EGFR (14). MDA-MB-468 and MCF-7 breast cancer cells were transfected with a kinase deficient c-Src cDNA in a pcDNA3.1 (Invitrogen) expression plasmid using 20μg of Lipofectin (GibcoBRL). Cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 20mM HEPES, 10 units/ml penicillin, 10 μg/ml streptomycin, and 400 μg/ml G418.

Western Immunoblot. Cells were lysed in RIPA buffer (150mM sodium chloride, 50mM Tris-base, pH 7.2, 1% deoxycholic acid, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 0.5% aprotinin, 12.5 μg/ml leupeptin, and 1mM sodium vanadate). 100 μg whole cell lysate protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Immobilon (Millipore). EGFR was visualized by the use of clone F4 mouse monoclonal antibody (Sigma) 1:1000. C-Src was visualized using purified 2-17 mouse monoclonal antibody (1μg/ml). Primary antibodies were detected using 1μCi/ml ^{125}I-labeled goat anti-mouse IgG (New England Nuclear).

Soft agar growth. Approximately 10^4 cells of MDA-MB-468 and MCF-7 or 10^5 cells of 10T1/2 clones were suspended in 0.5 % agarose containing DMEM, 2X Ham's F10, and 15% fetal bovine serum supplemented with 40ng/ml EGF for 10T1/2 clones or 10ng/ml EGF for the breast cancer cells. This suspension was layered over a 1% agarose plug in a 60mm dish and incubated for 2 weeks with media replenishment every 5 days. Colonies were stained with 1μg/ml iodonitrotetrazolium salt for 20 hrs. and counted.

RESULTS AND DISCUSSION

Generation of 10T1/2 mouse fibroblasts overexpressing EGFR and altered c-Src. To determine if various c-Src mutants could act to inhibit EGF-induced tumorigenicity, C3H10T1/2 mouse embryo fibroblasts that overexpress the EGFR and altered c-Src were created. An amphotropic helper virus was used to capture a hEGFR cDNA from a transfected NIH3T3 cell line provided by Dr. Doug Lowy. The hEGFR containing virus was then used to infect C3H10T1/2 clones that overexpressed either kinase deficient (K-), myristylation deficient (M-), or SH2 mutant (S-) c-Src. Individual clones were isolated by limiting dilution and tested for EGFR expression by western blotting (fig. 1). C-Src levels were comparable in each of the overexpressors (except for the S- mutant, which was expressed at a high level but turned over rapidly) and were ~20 fold over endogenous levels as determined by densitometry. hEGFR expression in the clones was estimated at 25,000 - 100,000 receptors per cell compared with ~10,000 endogenous receptors (27).

Growth in soft agar and tumor formation in nude mice of 10T1/2 clones overexpressing EGFR and altered c-Src. The morphology of the clones overexpressing EGFR and altered c-Src were significantly different in tissue culture dishes than the wild type overexpressors or vector-only controls. The M- c-Src/EGFR overexpressors (2AR) demonstrated a more transformed phenotype with a tendency to pile up on top of one another. The S- c-Src/EGFR overexpressors (155R) exhibited a fusiform morphology, while the K- c-Src/EGFR overexpressors (430R) were extremely flat and remained contact inhibited upon confluency. This observation suggested that the biological properties of the clones may be different. Therefore we tested the tumorigenic potential of the various clonal cell lines in soft agar assays and in nude mice. The 430R clones exhibited diminished anchorage independent growth in the presence and absence of EGF compared to the EGFR/*wt* c-Src double overexpressors (5HR) (table 1). However the K- c-Src did not ablate the function of the receptor comparing the number of colonies in soft agar of the 430R cells to that of the EGFR overexpressors. This suggests that *wt* c-Src exhibits a gain of function phenotype in the 5HR clones. It is important to note that the average size of the colonies arising from the 430R clones were much smaller than those arising from the EGFR overexpressors. The growth of tumors was completely inhibited in mice injected with 430R cells compared with NR or 5HR cells, suggesting that K- c-Src does have a dominant negative effect on tumor growth *in vivo*. In each assay more than one 430R clone was tested and yielded similar inhibition. In addition, we have shown that kinase deficient c-Src is still able to associate with the EGFR but that tyrosine-845 of the receptor is no longer phosphorylated when in complex with the defective c-Src. This could account for the inhibition of EGF-dependent soft agar growth by blocking binding

of critical substrates for the receptor and/or not allowing the hyperactivating phosphorylation on the receptor to occur. These results follow what we had hypothesized, that the kinase deficient c-Src could act in a dominant negative fashion in EGF-induced tumorigenicity as it did in EGF induced mitogenicity (28). Unexpectedly however, the myristylation deficient and SH2 mutant c-Src in the context of overexpressed EGFR potentiated EGF dependent and independent tumorigenicity (table 1) contrary to the mitogenic assay previously reported (28). This suggests that c-Src's role in mitogenic and tumorigenic signaling is divergent in these cells. It also tells us that these constructs can not be used to inhibit breast tumor cell lines as described in specific aim II. However, if the mechanism of transformation of these mutants can be elucidated, then we may be able to learn much about c-Src's role in tumorigenesis, thereby helping us to better target the molecule.

Generation of cell lines expressing myc-tagged N-terminal domains of c-Src. Since the kinase defective c-Src was able to inhibit tumorigenicity in the 10T1/2 cells, one or more of the amino terminal domains of c-Src is most likely causing the dominant negative effect. To see which domain of c-Src can inhibit tumorigenicity, several constructs were created for transfection into the 10T1/2 wild type double overexpressors, 5HR, or breast tumor cell lines. The unique, SH3, SH2, and the entire amino terminal domains (N-Term) were synthesized by PCR and cloned into Invitrogen's pcDNA3 myc-HIS vector. This puts the expression of the domains under the constitutive cytomegalovirus (CMV) promoter and adds a carboxy terminal myc tag for identification purposes. The constructs were also engineered to contain a portion of the amino terminal myristylation domain to allow for myristylation and proper subcellular localization. These constructs or a vector-only control were transfected into MDA-MB-468 breast tumor cell lines and individual clones were selected after G418 treatment. Individual clones of each myc-tagged domain have been isolated and are currently being assayed for growth in soft agar. Subcellular localization of the constructs has been determined by immunofluorescence after transient transfection or microinjection into 5HR11 10T1/2 wt double overexpressors and 468 cells. Each of the domains appears to have distributions similar to wild type c-Src, which includes perinuclear, plasma membrane, and endosomal distributions. The SH2 and N-Term domains also localize to focal adhesion structures. Experiments that combine BrDU incorporation or cell migration with microinjection of these constructs are currently underway.

Generation and characterization of MDA-MB-468 and MCF-7 cells overexpressing kinase deficient c-Src. Given the inhibitory effects of kinase deficient c-Src on 10T1/2 cells overexpressing the EGFR, we generated breast tumor cells that would overexpress the dominant negative form of c-Src. Kinase defective c-Src was synthesized by PCR and cloned into Invitrogen's pcDNA3.1 which is driven by a CMV promoter. The DNA was transfected into 468 and MCF-7 breast tumor cells, and individual clones were G418 selected. The clones were tested for expression of the kinase deficient c-Src by western blotting, and fold overexpression was estimated by densitometry (fig 2). The clones were tested for growth in soft agar and compared with vector-alone and parental cells. The kinase deficient c-Src overexpressors were able to dramatically inhibit anchorage independent growth of the breast tumor cells in the presence and absence of EGF in a dose dependent manner (table 2), supporting the findings in the 10T1/2 model system.

The decrease in soft agar colony formation upon EGF treatment in the 468 cells is due to the high level of EGFR overexpression and is similar to the observations seen in the human epidermoid carcinoma cell line, A431, which also expresses over 1 x 10^6 receptors/cell. A dose response of EGF treatment in soft agar growth of 468 cells is currently being performed. These results support the strategy designed above using individual amino terminal domains of c-Src to inhibit tumorigenicity of breast tumor cell lines.

RECOMMENDATIONS CONCERNING STATEMENT OF WORK

Significant strides have been made in specific aims I and II in the first year of this proposal. I predict that no problems that may arise could delay the completion of these aims after two years of work as designed in the statement of work, nor do I foresee the altering of any of the aims. The third specific aim will be started after the completion of the first two specific aims as planned.

CONCLUSIONS

The results indicate that kinase deficient c-Src decreases EGFR tumorigenicity in mouse fibroblasts and in human breast tumor cell lines, while the SH2 mutant and myristylation deficient c-Src potentiate EGF-dependent and independent colony formation. The kinase deficient c-Src diminished tumor formation in nude mice and colony formation both in the presence and absence of EGF in the mouse model system. The inhibition of tumorigenicity was correlated with the persistence of complex formation but the disappearance of the Y-845 phosphorylation on the receptor. The kinase deficient c-Src may be tying up binding sites on the receptor not allowing it to signal through its normal substrates. In addition, the disappearance of the hyperactivating phosphorylation at Y-845 suggests that c-Src's kinase domain is necessary for the phosphorylation to occur. The lack of this phosphorylation may also contribute to the lack of tumorigenicity of these cells since EGFR overexpressors without c-Src lack this phosphorylation and lack enhanced tumorigenic potential. In conjunction with the mouse model system, kinase deficient c-Src was able to act as a dominant negative in soft agar growth of MDA-MB-468 and MCF-7 breast cancer cell lines. These results strongly support the current strategy to use specific c-Src domains to inhibit breast tumor cell tumorigenicty. We are currently preparing a manuscript describing the kinase deficient c-Src effects on tumorigenicity. In contrast to the kinase deficient c-Src, the myristylation deficient and the SH2 mutant c-Src increase both the EGF-dependent and independent colony formation by unknown mechanisms.

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Appendix

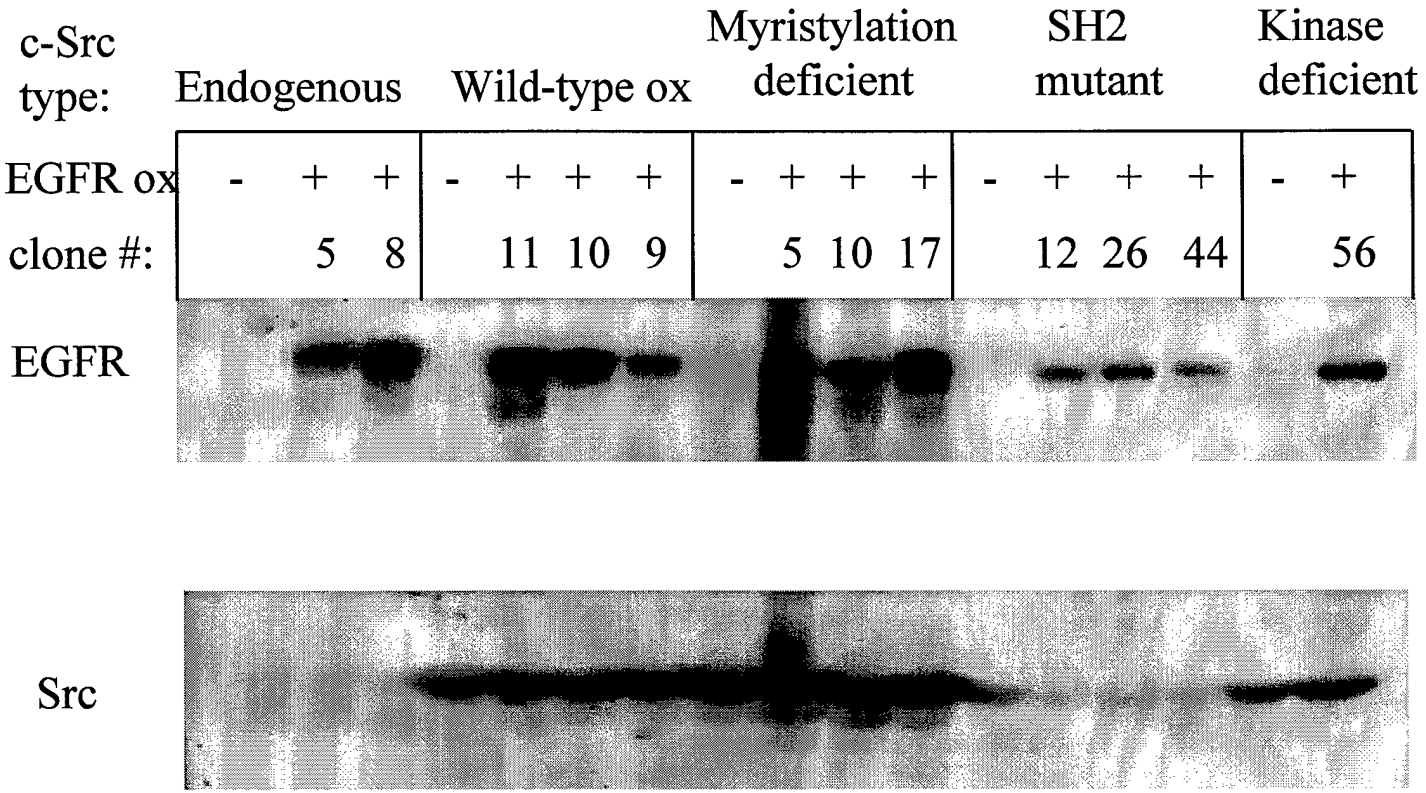
FIGURE LEGENDS

Figure 1 **Clones of C3H10T1/2 murine fibroblasts overexpressing EGFR and c-Src variants.** Lysate protein (100 μg) from the indicated clones was examined by Western immunoblot analysis after SDS/PAGE for EGFR and c-Src protein levels.

Table 1 **Soft agar colony formation of the 10T1/2 clones overexpressing EGFR and c-Src variants.** Values for number of colonies are the mean ± SEM obtained from a triplicate of one experiment plating 10^5 cells per plate. The experiment has been repeated in triplicate upon three separate clones of each c-Src variant with similar results.

Figure 2 **Overexpression of kinase deficient c-Src in MDA-MB-468 and MCF-7 breast cancer cells.** Lysate protein (100 μg) from the indicated clones was examined by Western immunoblot analysis after SDS/PAGE for EGFR and c-Src protein levels. The fold overexpression of kinase deficient c-Src compared to parental levels was determined by densitometry.

Table 2 **Inhibition of soft agar colony formation of MDA-MB-468 and MCF-7 breast cancer cell lines transfected with kinase deficient c-Src.** Values for number of colonies are the mean ± SEM obtained from a triplicate of one experiment plating 10^4 cells per plate. The experiment has been repeated three separate times with similar results.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Endogenous
Wild-type ox
Myristylation deficient
SH2 mutant
Kinase deficient
EGFR ox
- + + - + + + - + + + - + + + - +
clone #:
5 8 11 10 9 5 10 17 12 26 44 56
EGFR
Src

Figure 1

Cell Line	No. of colonies	
	No EGF	EGF
wt c-Src ox	6 ± 3	4 ± 2
Myristylation deficient c-Src ox	390 ± 40	350 ± 40
SH2 mutant c-Src ox	20 ± 10	50 ± 4
Kinase deficient c-Src ox	0	3 ± 3
EGFR ox	6 ± 3	230 ± 50
EGFR/c-Src ox	80 ± 5	470 ± 40
EGFR/Myristylation deficient c-Src ox	1000 ± 100	3200 ± 400
EGFR/SH2 mutant c-Src ox	2300 ± 300	3000 ± 400
EGFR/kinase deficient c-Src ox	0	290 ± 30

Table 1

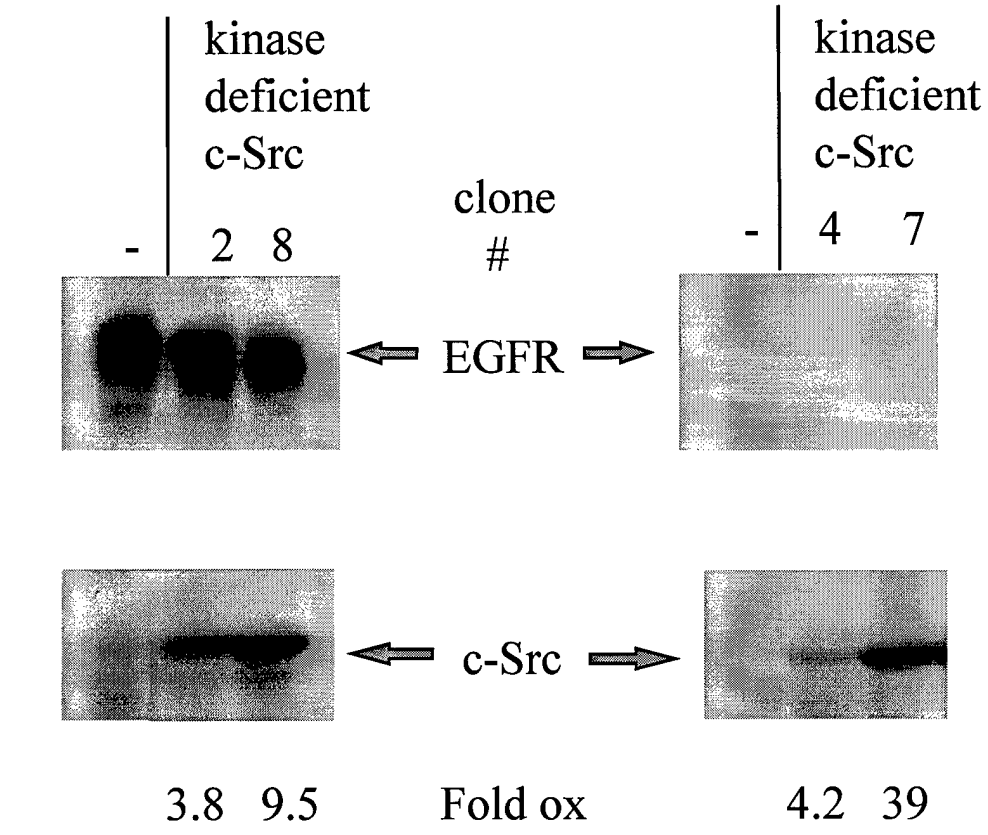
MDA-MB-468
MCF-7
kinase deficient c-Src
kinase deficient c-Src
clone #
- 2 8
- 4 7
EGFR
c-Src
3.8 9.5
Fold ox
4.2 39

Figure 2

Cell Line	No. of colonies	
	No EGF	**EGF**
MDA-MB-468	1028	252
MDA-MB-468 kinase deficient c-Src # 2	696	18
MDA-MB-468 kinase deficient c-Src # 8	152	3
MCF-7	448	503
MCF-7 kinase deficient c-Src # 4	243	182
MCF-7 kinase deficient c-Src # 7	42	95

Table 2